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RESEARCH ARTICLE

INHIBITION OF BACTERIAL PATHOGENS OF TROPICAL TASAR SILKWORM (*ANTHEREAE MYLITTA* D.) BY USING FOLIOSE LICHENS ISOLATED FROM SAL PLANT (*SHOREA ROBUSTA*).

Madhusudhan, K.N.*, Nisha-Kachhap, A.K. Sinha, Singh, G.P., Gupta, V.P., Naqvi, A.H. and Alok Sahay.

Department of Microbiology, Central Tasar Research and Training Institute, Piska Nagri, Ranchi-835303, Jharkhand, INDIA

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Email: kn.madhubiotech@gmail.com

Abstract: Bacterial diseases are causing considerable yield loss to tropical tasar silkworm (*A. mylitta* D.) during rearing. The presently common chemical disinfectants are being used for the control of bacterial pathogens. Lichens are potent antimicrobial compounds which are being exploited in different fields. Since, abundant presence of lichens was noticed in the tasar rearing plots, the present study was aimed to exploit the locally available lichens as antibacterial agents. The foliose lichen was collected from *S. robusta* plant and used in the present study. The lichens showed very promising results by inhibition pathogenic bacteria species infecting tropical tasar silkworm. The in vitro inhibition results revealed that, methanol and ethanol extract of lichens showed more zone of inhibition in comparison with other treatments and control. The separation of compounds by using TLC revealed the presence of different compounds at different RF value in both methanol and ethanol extract. The FTIR analysis of methanol and ethanol extract showed the presence of different functional groups in the extracts. Amino and alkene groups are responsible for the antimicrobial activity. Based on the results of the present study, the lichens can be used as potent biocontrol agent against bacterial pathogens infecting tropical tasar silkworm.

Key words: Tasar silkworm, Bacterial diseases, Lichens, Antibacterial activity, TLC, FTIR

INTRODUCTION:

Tasar silkworm, *Antheraea mylitta* D., is an economically important insect is mainly reared outdoor on its Primary food plants belonging to *Terminalia arjuna* (Arjun), *T.tomentosa* (Asan) and *Shorea robusta* (Sal). Several diseases affect tasar silkworm larvae during rearing. Pebrine, Virosis, Bacteriosis and Mycosis are the commonly prevalent diseases caused respectively by different pathogens viz., *Nosema* mylitta (Microsporidia), *Antheraea mylitta Cytoplasmic polyhedrosis* virus (AmCPV), different type of bacteria and *Penicellium citrinum* and *Paecilomyces varioti* (Fungus).

Tasar silkworm *Antheraea mylitta* Drury is susceptible to various bacterial pathogens that cause considerable yield loss during rearing. Death of worms due to bacteriosis occurs in every stage of its life cycle. However, loses in the larval stages is more visible which effects the crop, to the tune of 10-15% or sometimes more. The major types of pathogenic bacteria causing sealing of anal lips and rectal protusion in tasar silkworm were reported to be gram positive Bacillus and gram negative coccus (micrococcus).Chain type of excreta was *Microbacterium* (Sahay *et al*., 2000).

The secondary chemicals produced by lichens have attracted the attention of investigators for over 100 years, and information about their structure, biogenic origin and phylogenetic significance has accumulated steadily over this period (Lawrey, 1986). Lichens synthesize a great variety of secondary metabolites, many of which are unique. Developments in analytical techniques and experimental methods have resulted in the identification of about 1050 lichen substances (Molnar and Farkas, 2010). Most known lichen substances are usnic acid, phenolic compounds, anthraquinones, dibenzofurans, depsides, depsidones, depsones, triterpenes, gamma lactones and pulvinic acid derivatives and have a multiple biological activity: antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory and enzyme inhibitory (Lawrey, 1989). With this background, the present studied was aimed to exploit the naturally available lichens and lichen compounds for the control of bacterial diseases of silkworm particularly tasar silkworm.

Materials and Methods

Maintenance of pathogenic bacteria isolates

The pre-maintained pathogenic bacteria species in Central Tasar Research and Training Institute, Ranchi were inoculated to test tubes and flasks. The inoculated test tubes and flasks were kept at room temperature for 48hrs and observed for the bacterial growth.

Collection of lichens from tasar host plants:

The tasar host plants such as *Terminalia arjuna* (Arjun), *Terminalia tomentosa* (Asan) and *Shorea robusta* (Sal) plants having abundant lichens population were selected. The foliose lichens were collected with the help of knife along with small pieces of bark tissue. The bark tissue was removed from the lichens by using knife. The pure lichens were used for the further experimentation (Figure 1).

Fig. 1. The photographs of Foliose lichens present on *Shorea robusta* plant.

Preparation of lichens extract:

The powdered lichen (25g) was wrapped in 8x6 cm cylindrical pouch (Whatmann filter paper grade 1) and kept inside the extractor arm of the Soxhlet apparatus. A series of solvents (Chloroform, Ethanol, Methanol and water) were used for the extraction based on their polarity and each extraction was carried out at the specific boiling temperature for a period of 48hrs for the complete extraction of secondary compounds. The final filtrate of each of the extraction was concentrated using rotary evaporator.

Determination of antimicrobial activity

Each Pathogenic bacterial suspension was prepared in sterilized water, and the initial concentration of bacteria was adjusted to approximately 10^8 colony-forming units (CFU)/ml. Suspension of microbial cultures was inoculated on the entire surface of the Nutrient agar media in a Petri plate using sterile swab sticks. The sterile discs of diameter 6 mm were impregnated with lichen extract solutions (0.1 mg/mL and 0.2 mg/mL) and placed onto the cultured Nutrient agar agar plates. Inoculated plates were incubated at 37°C for 24 hrs. On the second day, plates were read by taking measurement of zone of inhibition around each disc. The diameter of zone of inhibition of bacteria was recorded in millimeters. Pure acetone, methanol, and ethanol were taken as negative control, whereas commercial Gentamicin and Ceftriaxone were used as positive control. Gentamicin was taken as positive control for gram positive bacteria and Ceftriaxone was used for gram-negative bacteria. The assay was done in triplicates and checked with the control plate.

Characterization of compounds from promising solvent extract:

Thin layer chromatography

The crude residue obtained from the cold as well as hot extracts were subjected to thin layer chromatography to check the present of different compounds. Various ratios among these solvents were used as a mobile phase. The suitable combination of solvents Methanol: Choloroform in the ratio of 9:1 was found to be effective in separating compounds.

FTIR analysis of Crude extracts of lichens

The FTIR analysis of the methanol and ethanol extracts was carried out by outsourcing (Central Instrumentation Laboratory, BIT Mesra, Ranchi). All the spectra were measured in the spectral range of 4000-450cm^{-1}.

Results

Studies on antimicrobial activity of lichen extracts on pathogenic bacteria species.

The *in vitro* inhibition of pathogenic bacteria using the lichen extracts showed the promising results. The all the extracts of lichen showed the activity against all the three tested bacterial pathogens of tropical tasar silkworm. Among the extracts treated Methanol and Ethanol extracts showed more zone of inhibition in comparison with other treatments and standard (Ampicilin) (Table 1) (Fig. 2, 3 & 4).

Characterization of Lichen compounds

Thin Layer Chromatography of lichen compounds

Based on the promising results of antimicrobial activity, both the methanolic and ethanolic extracts were used for the separation of compounds using thin layer chromatography (TLC). Five compounds were noticed in both methanolic and ethanolic extracts based on Retention factor (RF Value) (Table 2) (Fig. 5).

FTIR results:

The FTIR results showed that different functional groups present in both methanol and ethanol extracts of lichens. Among them, amino and alkene group of compounds responsible for the antimicrobial activity against bacterial pathogens infecting tropical tasar silkworm (Table 3 & 4) (Fig. 6 & 7).

Table 1. Estimation of Antibacterial activity

Pathogens	Methanol	Ethanol	Chloroform	water	Standard
Micrococcus sp.	10.1±0.75[a]	9.5±0.47[a]	6.7±0.52[c]	6.1±0.57[c]	9.9±0.51[b]
Microbacterium sp.	8.9±0.59[c]	9.3±0.79[b]	7.9±0.50[a]	6.9±0.75[a]	9.1±0.67[c]
Serratia sp.	9.5±0.57[b]	8.9±0.33[c]	7.2±0.37[b]	6.7±0.37[b]	10.1±0.53[a]

Table 2.The retention factors of compounds of methanolic and ethanolic extracts.

Compound	RF Value	
	Methanol	Ethanol
1	0.216	0.31
2	0.416	0.39
3	0.566	0.689
4	0.75	0.756
5	0.88	0.797

Table 3.Functional group analysis of methanolic extract of lichen.

Absorbance	Functional group
3471.87	Aromatic amine & Hydroxyl group
1411.89	Alkene group, Alcohol & hydroxyl group, Carbonyl compound, heteroxy compound.
1315.45	Alcohol and hydroxyl compound, Amino compound, carbonyl compound, heteroxy compound.
1033.85	Amino group, alkane group, heteroxy compound, common inorganic ion.
948.98	Alkane Group (Methylene group), Inorganic ion.
709.80	Aromatic (Aryl) group, Alcohol and hydroxyl compound, Alkane group.

Table 4.Functional group analysis of ethanolic extract of lichen.

Absorbance	Functional group
3545.16	
1435.04	Alkyl group (methyl), inorganic ion (carbonate ion).
1319.31	Alcohol& hydroxyl group, Aromatic amino, carbonyl compound, hetero-oxy compound.
1203.58	Saturated aliphatic alkyne group (methyne group), Aromatic ring (aryl) group, Amine
952.84	Saturated aliphatic (alkane/alkyl) group, Aromatic ring (aryl), hetero-oxy compound, Inor-
767.67	Aromatic ring (aryl) group, Aliphatic organohalogen group (chloro), hetro-oxy compound,

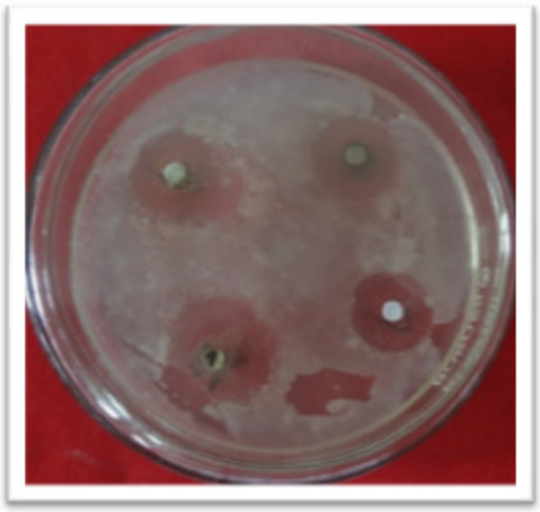

Fig. 2. The antibacterial activity of different solvent extracts on *Micrococcus* species (Rectal protrusion)

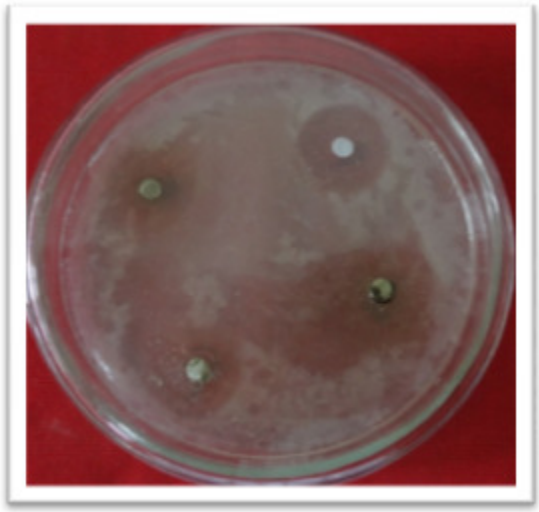

Fig. 3. The antibacterial activity of different solvent extracts on *Microbacterium* species (Sealing of Anal Lips)

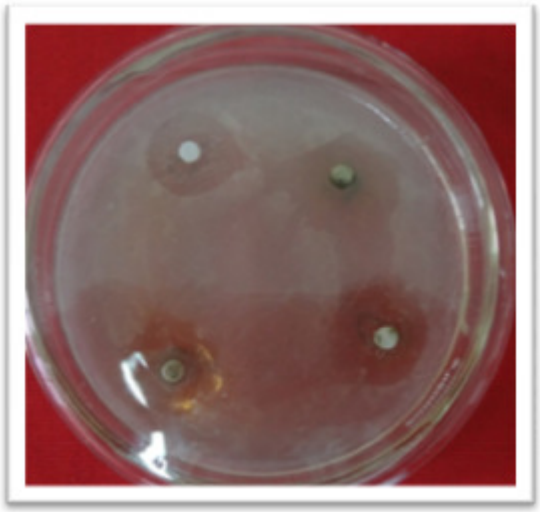

Fig. 4. The antibacterial activity of different solvent extracts on *Serratia* species (Chain type excreta)

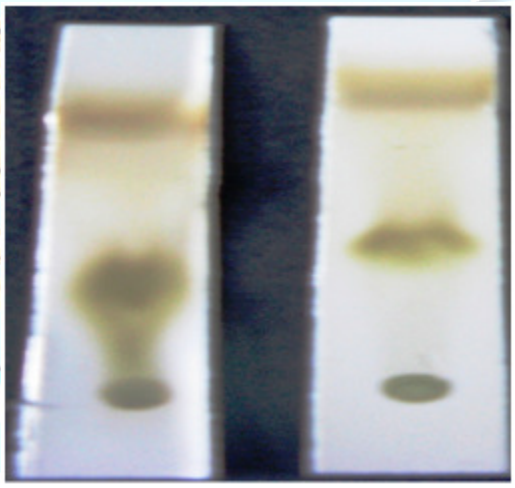

Fig 5. The TLC profile of methanolic and ethanolic extracts of lichens.

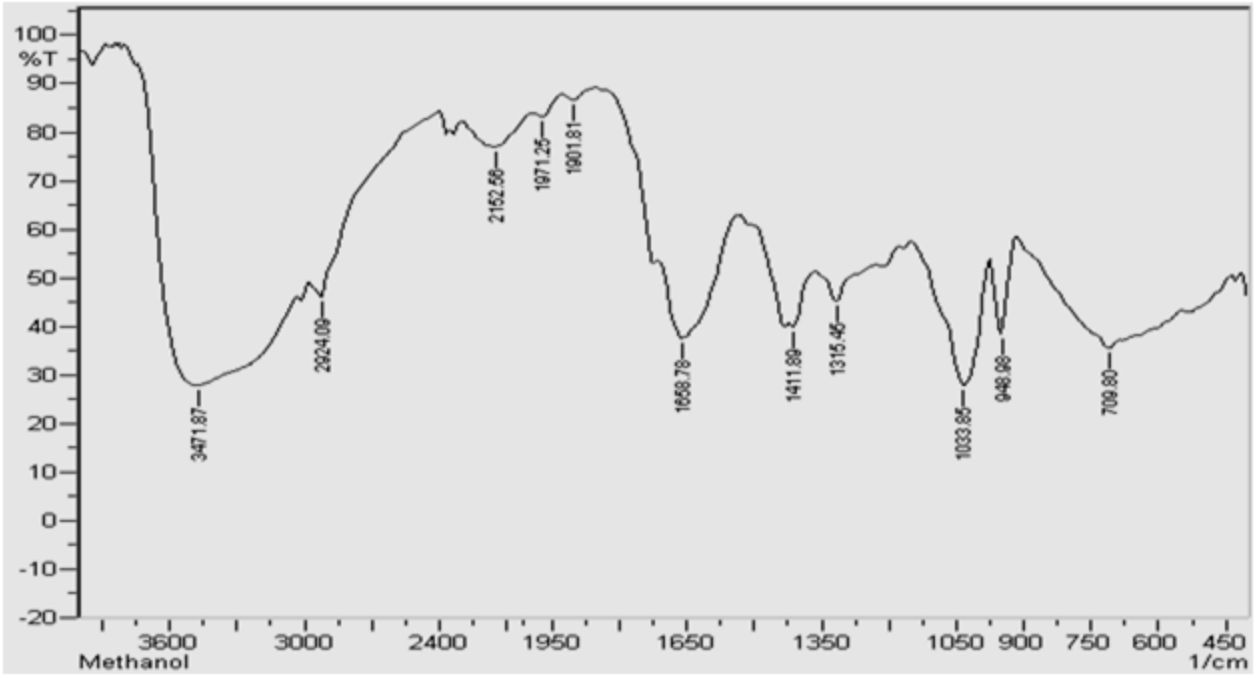

Figure 6 . FTIR profile of Methanolic extract of the lichen.

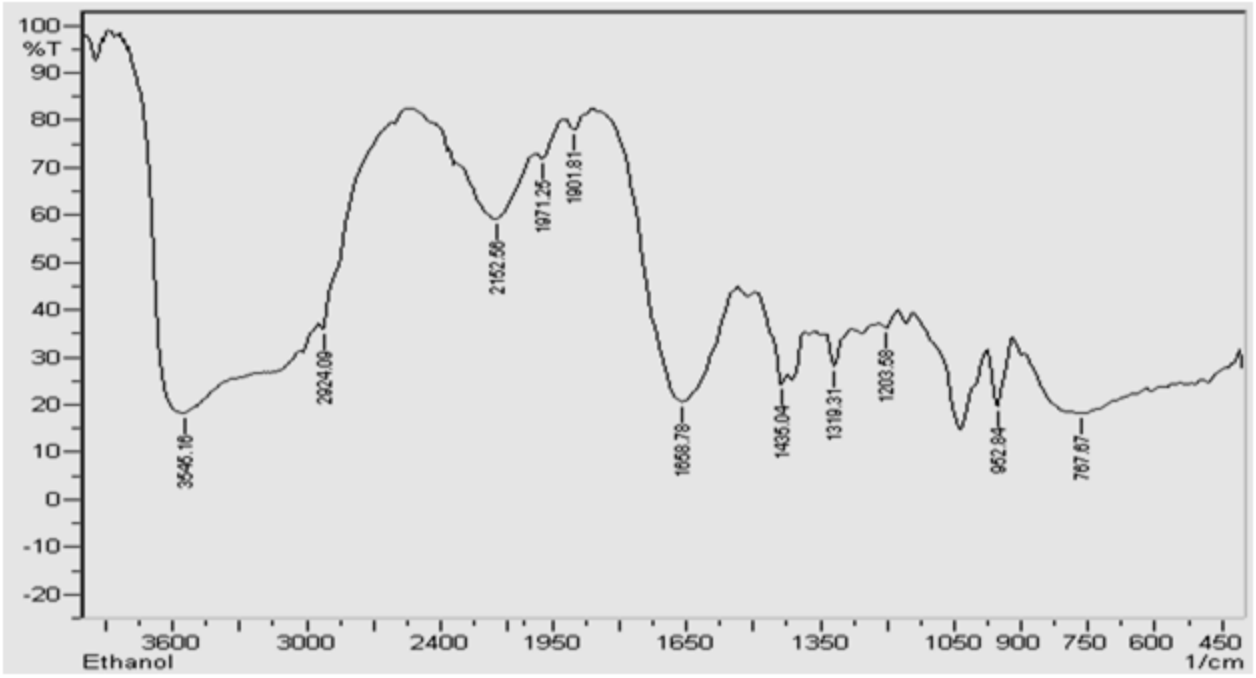

Figure 7. FTIR profile of Ethanolic extract of the lichen.

Discussion

The tasar culture is being practiced in different states of the central and north eastern states of India. The silk-worm is being affected by different pathogens. Among them, bacterial pathogens are very important disease. The bacterial pathogens imparts different symptoms such as Rectal protrusion (RP), Sealing of Anal Lips (SAL) and Chain Type Excreta (CTE) on the silkworm larva which are causing considerable yield loss upto 20-30% during rearing period (Sahay *et al*., 2000).

Lichens synthesize a great variety of secondary metabolites, many of which are unique. Developments in analytical techniques and experimental methods have resulted in the identification of about 1050 lichen substances (Molnar and Farkas, 2010).

Most known lichen substances are usnic acid, phenolic compounds, anthraquinones, dibenzofurans, depsides, depsidones, depsones, triterpenes, gamma lactones and pulvinic acid derivatives and have a multiple biological activity: antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory and enzyme inhibitory (Lawrey , 1989).

In the present study also, the antibacterial activity of lichen extracts was studied against the pathogenic bacteria species infecting tropical tasar silkworm. The results showed that, the extracts have the antibacterial activity which can be exploited in the tasar culture field (Table 1). The TLC is being used to analyze the number of compounds in the solvent extracts of biological materials. In our study also, TLC has been employed to separate the promising bioactive compounds from the both methanol and ethanol solvent extract of lichens. The functional group analysis of methanolic and ethanolic extract of lichens showed that, different functional groups are present. Among the functional groups, amino and alkenes showed the antimicrobial activity against pathogens (Table 3 & 4). Similarly, Behera *et al.* (2007) reported that acetone, methanol and petroleum extracts of lichen *Usnea ghattensis* were effective against different bacterial species.

Based on the results of the present research, the lichens can be used as potent biocontrol agent against pathogens infecting tropical tasar silkworm which leads to enhanced productivity of tasar.

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